

Molecular cloning and expression of glycogen synthase kinase-3/Factor A

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Glycogen synthase kinase-3 (GSK-3) is a protein-serine kinase implicated in the hormonal control of several regulatory proteins including glycogen synthase and the transcription factor *c-jun*. Two classes of rat brain cDNA for this enzyme have been isolated termed GSK-3 α and GSK-3 β . The α -type encodes a 51 kd polypeptide, the sequence of which includes all of the tryptic peptides determined by protein sequence analysis of purified skeletal muscle GSK-3. The novel β -type cDNA has the potential to encode a 47 kd protein with 85% amino acid identity to GSK-3 α . The two types of cDNA are the products of distinct genes as determined by genomic organization and nucleic acid sequence analysis. Both α and β clones exhibit kinase activity when expressed in COS-1 cells and type-specific antibodies to GSK-3 α and β detect proteins of 51 and 47 kd, respectively, in a variety of rat tissue extracts, with highest levels of both in brain. Partial purification of GSK-3 activity from bovine brain results in the isolation of active α and β proteins. The physiological importance of these two proteins in cellular signal transduction is discussed.

Key words: cDNA/*c-jun*/kinase/PCR/serine

Introduction

Studies of the normal role of proto-oncogenes in cell growth and differentiation have implicated this class of proteins in a number of signalling pathways from growth factor receptors at the plasma membrane to transcription factors in the nucleus. In an effort to identify the mechanisms via which signals at the cell surface are transmitted to nuclear transcription factors, we have been studying the processes by which two nuclear proto-oncogenes *c-myc* and *c-jun* are normally regulated (Klempnauer *et al.*, 1982; Boyle *et al.*, 1983; Bohmann *et al.*, 1987; Angel *et al.*, 1988). Both proteins act as transcription factors: *c-jun* is a component of AP-1, mediating gene activation by agents such as phorbol esters and insulin (Lee *et al.*, 1987; reviewed in Mitchell and Tjian, 1989); *c-myc* regulates gene expression in cells of the myeloid lineage (Ness *et al.*, 1989). *In vivo*, these two proteins are phosphorylated on serine and threonine residues and in an attempt to identify the protein-serine kinase(s) responsible for their phosphorylation, we surveyed a selection of characterized enzymes. Only one protein kinase, glycogen synthase kinase-3 (GSK-3), phosphorylated *jun* and *myb* exclusively at sites identified *in vivo* by phosphopeptide mapping (W. Boyle, T. Smeal, L. Defize, P. Angel, J.R. Woodgett, M. Karin and T. Hunter, in preparation).

Furthermore, the site phosphorylated in *c-jun* by GSK-3 is regulated in cells; phorbol ester treatment causes specific dephosphorylation. *In vitro*, phosphorylation of *c-jun* by GSK-3 inhibits DNA binding, suggesting that agonist-induced dephosphorylation in cells is involved in *c-jun* activation (Boyle *et al.*, in preparation).

GSK-3 was first identified as one of the protein kinases that phosphorylates glycogen synthase, the rate-limiting enzyme of glycogen deposition (Embi *et al.*, 1980; Hemmings *et al.*, 1982). Insulin causes site-specific dephosphorylation of glycogen synthase specifically at residues targeted by GSK-3, causing activation of the enzyme (Parker *et al.*, 1983; Cohen, 1985; Cohen *et al.*, 1985; Poulter *et al.*, 1988). GSK-3 is identical to Factor A (F_A), the activator protein of an inactive MgATP-dependent form of a broad specificity protein-serine/threonine phosphatase, termed phosphatase-1 (Vandenhede *et al.*, 1980; Hemmings *et al.*, 1982; reviewed in Cohen, 1989). Since this phosphatase dephosphorylates substrates of GSK-3, this protein kinase potentially catalyses opposing reactions. However, the physiological significance of F_A activity is presently unclear (Cohen, 1989).

Agonist-induced dephosphorylation of *c-jun* and glycogen synthase could occur by either inhibition of GSK-3 or activation of a protein phosphatase (or both). Although GSK-3/F_A has been previously purified by several groups (Hemmings *et al.*, 1982; Woodgett and Cohen, 1984; Tung and Reed, 1989), its mode of regulation *in vivo* is unknown. As a first step in assessing the role of GSK-3 in hormonal regulation we have isolated cDNA clones of this protein kinase, revealing it to comprise a multigene family. The structure, expression and enzymatic function of two GSK-3 proteins are described, and their potential involvement in the mechanism of action of insulin and regulation of proto-oncogenes is discussed.

Results

Peptide sequence determination and amplification of GSK-3 cDNA

Purification of GSK-3 from 2 kg rabbit skeletal muscle resulted in the isolation of 25–30 μ g of a single polypeptide of 51 kd as described previously (Woodgett, 1989). Reverse-phase HPLC of a tryptic digest of this material resolved several peptides which, following rechromatography, were subjected to peptide sequence analysis. In all, 11 peptides yielded information. A further peptide sequence was generated from digestion with *Staphylococcus aureus* V8 protease. Comparison of the peptide sequences with the Swissprot database (September, 1989) revealed no exact matches, although visual alignment of two peptides (DIKPQN... and VLGTPPT) revealed similarity to sequences within other protein-serine kinases (Hanks *et al.*, 1987).

Whilst the peptides were being sequenced, a six amino

2432

the high degeneracy of the sense oligonucleotide (62 000-fold), a single fragment of 340 bp was amplified from both HeLa and rat brain cDNAs. The nucleotide sequences of three independent clones of these fragments contained the same potential open reading frame that, at the 5' end, corresponded to the C-terminal portion of the DIKPQN... peptide. The amino acid juxtaposing the sequence encoded by the 3' oligonucleotide was an arginine consistent with the cleavage specificity of trypsin. Furthermore, the VLGTPT peptide sequence was contained within the fragment and was preceded by a lysine residue; this confirms that the PCR product was derived from GSK-3 cDNA. The nucleotide differences between the brain and HeLa fragments were silent with respect to the amino acid sequence.

Since brain has been reported to be a rich source of GSK-3 (Tung and Reed, 1989), a rat brain cDNA library was screened with the brain PCR fragment and 10 positive plaques isolated. Nucleotide sequence analysis of these clones delineated two types of cDNA. Five of the isolates were derived from one gene and three of these contained an open reading frame of 483 amino acids, with the potential to encode a protein of 50.89 kd (Figure 1A). Within this predicted sequence were all of the purified peptides, preceded by lysine/arginine (tryptic peptides) or glutamic acid (V8

A smaller difference in the synthesis of the two clones was observed upon transient transfection of COS-1 cells. Immunoblotting with antibodies specific for GSK-3 α or β (see below) revealed the presence of a major 51 kd protein and a minor 59 kd protein in cells transfected with GSK-3 α DNA and a 47 kd protein in GSK-3 β transfectants (Figure 2B). Since the 59 kd band was immunologically related to GSK-3, it is likely to be derived from an upstream CTG codon in the GSK-3 α cDNA since there are no other ATG codons in the 5' untranslated region. Fractionation of cell lysates of monoS FPLC revealed a 3-fold increase in

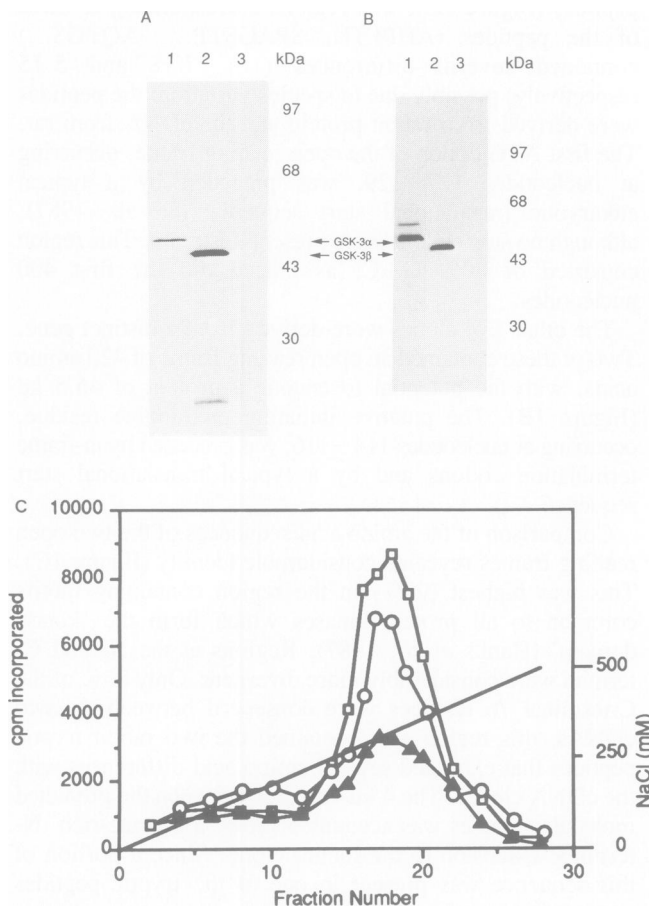


Fig. 2. Expression of GSK-3 proteins in reticulocyte lysates and COS-1 cells. In (A), rabbit reticulocyte lysates containing [35 S]methionine were programmed with SP6-transcribed cRNA from GSK-3 α cDNA (lane 1), GSK-3 β cDNA (lane 2) or water (lane 3). A fraction (10%) of the newly synthesized proteins were resolved on SDS-polyacrylamide gels (Laemmli, 1970) and detected by fluorography (-70°C , 6 h). In (B), detergent extracts of COS-1 cells transfected with GSK-3 α (lane 1), GSK-3 β (lane 2) or pmt-2 vector alone (lane 3) were electrophoretically separated, electroblotted onto PVDF membrane and detected with a mixture of anti-GSK-3 α and β antibodies as described in Materials and methods. In (C), extracts of COS-1 cells transfected with GSK-3 α (circles), GSK-3 β (squares), or pmt-2 alone (triangles) were subjected to FPLC chromatography on monoS beads and fractions assayed for glycogen synthase kinase activity.

glycogen synthase kinase activity in the α and β -transfected cells (Figure 2C). The lower-than-expected recovery of activity may be partially due to insolubility of a fraction of the expressed proteins in the COS-1 cells (data not shown).

Tissue distribution of GSK-3 α and GSK-3 β

Nucleic acid probes derived from the α and β cDNAs were used to detect expression of GSK-3 mRNA. Probes and hybridization/washing conditions were chosen that prevented cross-hybridization between the two gene products. The GSK-3 α probe detected a 2.5 kb RNA in all nine of the rat tissues examined: the GSK-3 β probe detected lower, but again ubiquitous, levels of 2.3 kb RNA (Figure 3A). Since the largest cDNA clones of α and β were 2.2 and 1.6 kb respectively, some of the 5' untranslated region of β cDNA is likely to be missing (even allowing for a poly(A) tail of ~ 200 nucleotides). While the size of the RNA detected by

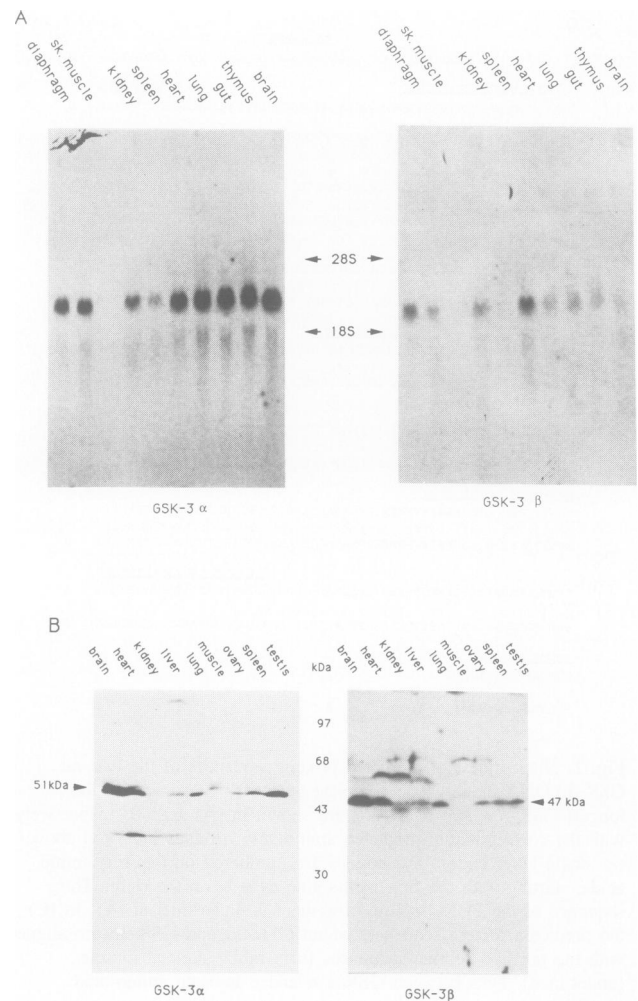


Fig. 3. Tissue distribution of GSK-3 RNAs and proteins. In (A), Northern blot analysis was performed sequentially using the same blot with probes derived from GSK-3 α (left panel) or GSK-3 β (right panel) as described in Materials and methods, using total RNA (15 μg) isolated from the indicated tissues. Arrows indicate the migration of 18S and 28S rRNA. In (B), 200 μg of protein extracted from the indicated tissues was immunoblotted with either antibodies directed against GSK-3 α (left panel) or GSK-3 β (right panel). Mol. wt markers are indicated.

the GSK-3 β probe was only slightly smaller than that of GSK-3 α , the relative levels of expression between different tissues was different, confirming that the β signal was not due to hybridization with GSK-3 α RNA. Standardizing for specific activity (see below), GSK-3 α RNA was 3- to 10-fold more abundant than that for GSK-3 β , depending on the tissue.

Polyclonal antibodies were raised against bacterially expressed portions of the two types of GSK-3. Despite the presence of common sequences, the antisera exhibited a degree of specificity for their respective proteins (see below). The relative differences in RNA expression in each tissue were not conserved at the protein level as judged by immunoblotting of tissue extracts (Figure 3B). A 51 kDa protein was specifically detected by antiserum raised against a GSK-3 α fusion protein, whereas a 47 kDa protein was detected by antiserum directed against GSK-3 β . The disparity between the comparative levels of RNA and protein was exaggerated in brain; a 10:1 ratio of α : β RNA being reduced

to an α : β protein ratio of 1.5:1. It should be noted that the high concentration of actin (45 kd) in skeletal muscle extracts distorts the migration of other proteins in the 40–50 kd mass range. Immunoreactive staining for GSK-3 α and β is present in these extracts but is diffused by the actin. However, the total absence of GSK-3 β from preparations of skeletal muscle GSK-3 is somewhat surprising. It is possible that the β RNA is not translated in muscle or that the protein is lost during purification (but see below).

Copurification of GSK-3 α and β from bovine brain

Since brain appeared to be a rich source of GSK-3 β (as well as GSK-3 α), GSK-3 activity was partially purified from bovine brain by sequential chromatographies on anion and cation exchangers, phosphocellulose, heparin–Sephacrose and gel filtration (Figure 4). The procedure used was similar to that reported by Tung and Reed (1989), who purified F_A activity from bovine brain. The preparation exhibited a specific activity of 0.35 μ mol/min/mg using glycogen synthase as a substrate. Homogeneous skeletal muscle GSK-3 (51 kd) has a specific activity of 2.1 μ mol/min/mg, suggesting ~17% purity for the brain preparation.

Incubation of partially purified GSK-3 from brain and skeletal muscle with [γ - 32 P]ATP allowed autophosphorylation of the protein kinase subunit(s). As expected, in the muscle preparation [32 P]phosphate was incorporated into a smeared band of 50–56 kd (Figure 5, lane D). At stoichiometries above 1 mol phosphate/mol 51 kd band, autophosphorylation retards the mobility of GSK-3 in SDS gels (Woodgett and Cohen, 1984). In the brain preparation two bands of ~51 kd and 47 kd were labelled to an equimolar degree (Figure 5, lane B). The brain and skeletal muscle enzymes both phosphorylated a 68 kd bacterially synthesized *myb* protein that is an effective substrate for GSK-3 (Figure 5, lanes A and C) (W.Boyle, J.R.Woodgett and T.Hunter, in preparation).

Immunoblotting of a 20% pure GSK-3 preparation from rabbit skeletal muscle with antibodies to both GSK-3 α and β revealed a predominant band of 51 kd and a minor band of 48 kd (Figure 5, lane F). These two components were most efficiently detected by the α -specific antibodies (not shown). The muscle preparation thus contains GSK-3 α (51 kd) and a related protein (48 kd, termed GSK-3 α') but none of the β form. The brain GSK-3 preparation contained a similar ratio of the GSK-3 α and α' bands of 51 and 48 kd respectively (Figure 5, lane E). However, this preparation also exhibited a major band of 47 kd. When probed with the α and β antisera separately, the 51 kd protein was selectively detected by the α -specific antiserum whereas the 47 kd band cross-reacted with the β -specific antibody (Figure 5, lanes G–J). Since the ratio of α to β proteins in the purified preparation was similar to that in brain extracts (not shown), the two proteins were copurified with similar recoveries, suggesting similar physico-chemical properties. The origin of the GSK-3 α' protein is, at present, unclear. Since it does not appear to be present in immunoblots of whole tissue, it may be a degradation product of GSK-3 α . However, this protein is of low abundance compared with GSK-3 α thus impairing detection and may be enriched with respect to GSK-3 α upon purification. It has been consistently observed in all stages of purification of GSK-3 from muscle and brain tissue and thus may possibly represent a third form of the enzyme (see below).

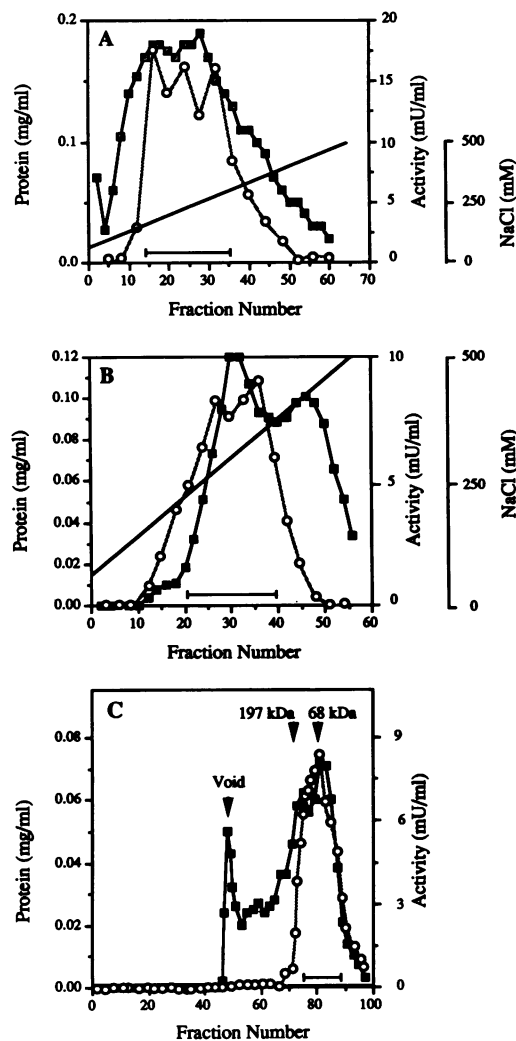


Fig. 4. Partial purification of GSK-3 from bovine brain. Chromatography of GSK-3 on: (A), phosphocellulose; (B), heparin–Sephacrose; (C), Sephacryl-S300 HR. For details see Materials and methods; glycogen synthase kinase activity (circles), protein (squares). Bars represent the pooled fractions. In (C), the elution positions of the void volume, phosphorylase b (197 kd), and BSA (68 kd) are indicated.

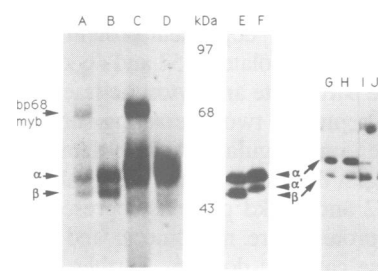


Fig. 5. Autophosphorylation and immunoblotting of purified preparations of GSK-3. Partially purified GSK-3 from bovine brain (lanes A and B) or skeletal muscle (lanes C and D) were incubated in the presence of [γ - 32 P]ATP. Bacterially synthesized *myb* (68 kd) was included in the incubations in lanes A and C. Purified brain (lane E) or muscle (lane F) GSK-3 was immunoblotted with a mixture of GSK-3 α and β antibodies. Immunoblotting of brain GSK-3 was performed with anti-GSK-3 α (lanes G and H) or anti-GSK-3 β antibodies (lanes I and J). Mol. wts are indicated.

Genomic blotting suggests the existence of further GSK-3-like genes

Hybridization patterns of rat DNA with probes derived from the α and β cDNAs at high stringency were consistent with single copy genomic content (Figure 6, panels A and B). However, low stringency screening with the GSK-3 α probe detected an array of lower intensity bands (Figure 6C). Some of these bands corresponded to fragments of similar gel mobility to GSK-3 β (indicated by arrowheads), but most were distinct. This low level hybridization is possibly to other genes encoding GSK-3-like proteins and might also explain the presence of the 48 kd α' protein in the partially purified preparations from muscle and brain (Figure 5).

Discussion

The existence of multiple forms of GSK-3 is not unusual given the multiplicity of other molecules involved in signal transduction, such as the seven protein kinase Cs and five or more phospholipase C enzymes (Nishizuka, 1988; Rhee *et al.*, 1989). While preparations containing equal amounts of the α and β forms behave similarly to pure α with respect to substrate specificity (that is, phosphorylation of glycogen synthase, *myb* and *jun*; data not shown), there are, as yet, no preparations of GSK-3 β totally devoid of GSK-3 α (β -transfected COS-1 cells, for example, also express endogenous GSK-3, Figure 2C; see below). Studies of the protein kinase C family have revealed subtle differences, such as in cofactor interactions and substrate preferences, that become detectable only upon separation of the various forms of either purification or their individual expression in heterologous cells (Parker *et al.*, 1989). Expression of the two GSK-3 genes in baculovirus-infected insect cells might, similarly, reveal differences. Unlike the protein kinase C genes, GSK-3 α and β appear to be expressed in most tissues (Figure 3B). However, in a survey of mammalian cell lines, a small proportion were found that express only GSK-3 α , such as A431 epidermoid carcinoma cells (data not shown). To date, no lines exclusively expressing GSK-3 β have been found.

Although this is the first demonstration of the existence of more than one type of GSK-3, Tung and Reed (1989) purified GSK-3 from bovine brain by following its ability to activate the MgATP-dependent protein phosphatase (F_A activity). This group isolated a 52 and 46 kd protein doublet from both the particulate and cytosolic fractions which were suggested to represent two interacting subunits of GSK-3; one catalytic, one regulatory. The data presented here strongly suggest this not to be the case: rather, if, as seems likely, the 52 and 46 kd proteins represent GSK-3 α and β , the two proteins are monomeric and each contains a protein kinase catalytic domain. Further, GSK-3 α clearly has activity in the absence of GSK-3 β (Woodgett and Cohen, 1984; Woodgett, 1989; this report). It is conceivable that the elevation in total GSK-3 activity in COS-1 cells expressing GSK-3 β is due to it activating endogenous GSK-3. Thus unequivocal evidence for GSK-3 β having kinase activity must await its expression and purification to homogeneity, which is in progress using the baculovirus system. The similarity in the catalytic domains between the α and β forms suggests they phosphorylate similar proteins; thus GSK-3 β is also likely to display F_A activity. The

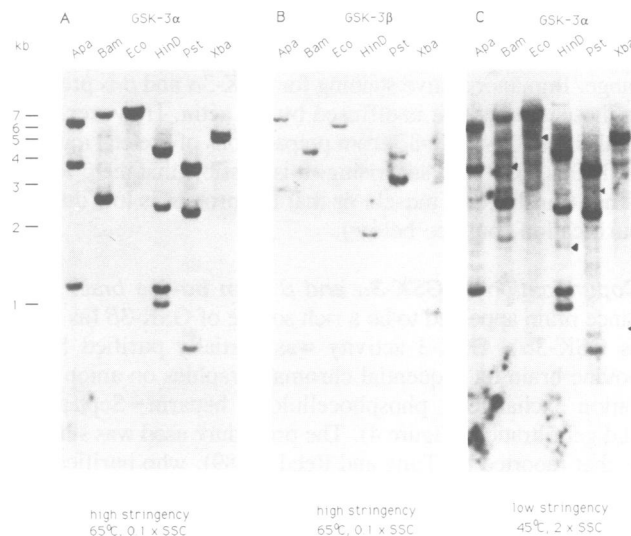


Fig. 6. Southern blot analysis of GSK-3. Rat genomic DNA (15 μ g) was fragmented, electrophoretically separated, transferred to Zetabind and hybridized with random-primed probes derived from GSK-3 α (panels A and C) or GSK-3 β (panel B) using the indicated washing conditions (see Materials and methods for probe details). Exposure was 6 h at room temperature (panel C) or 24 h at -70°C (panels A and B). Arrowheads in panel C indicate positions of GSK-3 β fragments.

biological significance of this activity has recently been drawn into question since no phosphate has been detected *in vivo* at the site on the phosphatase subunit, termed inhibitor-2, phosphorylated by GSK-3 (Cohen, 1989). The ability to increase or reduce GSK-3 RNA levels in cells using inducible promoters and antisense DNA or to introduce purified protein (by micro-injection or scrape-loading) may help clarify this matter.

As mentioned above, GSK-3 is present in both particulate and soluble fractions within cells. Indeed, the extraction conditions for purification from COS-1 cells required the presence of non-ionic detergent, otherwise $\sim 70\%$ of the protein is associated with the particulate fraction. Preliminary immunofluorescence and immunoblotting data indicate association of a fraction of GSK-3 α and β with the plasma membrane (data not shown). Such a localization is consistent with a role for GSK-3 in transduction of signals from the cell surface. The ubiquitous expression of the enzyme suggests that it has roles other than in glycogen metabolism. Studies are in progress to monitor the subcellular localization of this protein kinase upon hormonal stimulation as well as changes in its phosphorylation state.

A major impetus for molecular characterization of GSK-3 derived from recent observations implicating this protein kinase in the hormonal regulation of the *c-jun* proto-oncogene (W.Boyle, T.Smeal, L.Defize, P.Angel, J.R.Woodgett, M.Karin and T.Hunter, in preparation). Phorbol esters stimulate the specific dephosphorylation of *c-jun* at a site(s) phosphorylated by GSK-3 *in vitro*. In the case of *c-jun* this results in de-inhibition of DNA binding and thus transcriptional activation via *c-jun* homodimers (W.Boyle *et al.*, in preparation); these experiments were performed with purified GSK-3 α . While the ability of GSK-3 β to phosphorylate *c-jun* has yet to be formally addressed, the finding that many cells express two forms of GSK-3 complicates experiments in the evaluation of the role of this

protein kinase in *c-jun* regulation. However, availability of nucleic acid probes and antibodies will help in the elucidation of the molecular mechanisms by which these protein kinases are regulated in cells, as well as the processes which they, in turn, regulate.

Materials and methods

Purification of skeletal muscle GSK-3

GSK-3 was purified from rabbit skeletal muscle essentially as described (Woodgett, 1989), except that following chromatography on monoS, the peak of GSK-3 activity was subjected to reverse phase HPLC using a C4 column (2.1 mm, wide-pore, Vydac) in 0.1% trifluoroacetic acid and eluted with acetonitrile (at ~35%).

Proteolysis of muscle GSK-3 and separation of peptides

The purified protein (20–25 µg) was fragmented either by trypsin (5% by weight, Worthington, TPCK-treated) at 37°C for 18 h, or with *S.aureus* V8 protease (5% by weight, Boehringer Glu-C protease) using standard conditions. The resulting peptides were resolved on a C8-RP300 reverse-phase column in 50 mM ammonium acetate pH 6.8 with acetonitrile elution (0–50%) (Tempste *et al.*, 1986). Single peaks were rechromatographed on a small scale column of the same media but using 0.08% trifluoroacetic acid, pH 2.1. Peptides (25–70 pmol) were sequenced using an Applied Biosystems 477A sequencer (Hewick *et al.*, 1981). In total, peptide sequence data were obtained from four different preparations of GSK-3.

Amplification of GSK-3 cDNA

Two oligonucleotides corresponding to two of the tryptic peptide sequences (H/Y-R-D-L/I-K-P-E/Q-N and E-M-N-P-N-Y) were designed as primers for the polymerase chain reaction (PCR). Amplification was performed with *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus) according to the suppliers instructions (Mullis *et al.*, 1986). 30 cycles consisting of denaturation at 93°C for 1 min, annealing at 50°C for 2 min, and polymerization at 70°C for 5 min were performed using 1 µg of HeLa cDNA (in Okayama and Berg vector linearized with *Bam*HI, complexity of 1×10^6 ; Hanks, 1987) or 1 µg of rat brain cDNA (in λ ZAPII vector, complexity 2×10^6 , Stratagene) in a programmable heating block. The amplified products of 340 bp were gel-purified, ligated to *Eco*RI adaptors (Promega), and inserted into Bluescript (Stratagene) for sequence analysis.

Isolation of full-length clones

A random-primed probe (5×10^8 c.p.m./µg, 10^6 c.p.m./ml; Feinberg and Vogelstein, 1983) was generated from the 340 bp PCR fragment and used to screen 10^6 recombinants of a rat brain cDNA library in a λ ZAPII vector (Stratagene). Hybridization conditions were: $5 \times$ SSPE, 100 µg/ml sonicated and denatured salmon sperm DNA, $5 \times$ Denhardt's, 1% SDS, 50% formamide at 42°C for 18 h. The filters were washed with $2 \times$ SSPE, 0.1% SDS at 42°C for 2 h, followed by $0.5 \times$ SSPE, 0.1% SDS at 55°C for 2×20 min and autoradiography for 18 h at -70°C with intensifying screens. Ten positive plaques were purified, pBluescript plasmids rescued by super-infection with R408 helper phage (Short *et al.*, 1988), and subjected to DNA sequence analysis on both strands by use of exonuclease III-nested deletions, restriction fragment cloning into M13 and specific oligonucleotide primers. The 10 clones fell into two classes: λ C (1.2 kb), λ D (1.6 kb), λ G2 (2.2 kb), λ H (1.4 kb), λ N5 (2.2 kb) were derived from one gene and λ I (1.5 kb), λ J (1.6 kb), λ K1 (1.6 kb), λ K2 (1.1 kb) and λ N1 (1.5 kb) from a second gene.

DNA and RNA analysis

Total RNA was isolated from various rat tissues (Chirgwin *et al.*, 1979), and 10 µg of each electrophoretically separated on formaldehyde-agarose gels and transferred to GeneScreen (Du Pont). Blot hybridizations and washes were performed as described above using a 0.75 kb *Bam*HI fragment from clone λ J (GSK-3 β , nucleotide 681–3' polylinker). Following autoradiography, the membrane was stripped and hybridized with a 1 kb *Eco*RI fragment from clone λ D (GSK-3 α , nucleotide 1180–3' polylinker). For Southern blots, 15 µg rat genomic DNA was digested with the indicated restriction enzymes, electrophoretically separated, transferred to Zetabind (CUNO) and hybridized according to the manufacturers' protocol with the probes used for the RNA analysis. Low stringency washing was for 2 h with five changes of $2 \times$ SSPE, 0.1% SDS at 42°C. The high stringency washes contained $0.1 \times$ SSPE, 0.1% SDS at 65°C for 2×20 min.

In vitro transcription and translation

An *Eco*RV–*Bam*HI fragment of GSK-3 α (5' polylinker–3' polylinker containing entire cDNA) and a *Rsa*I–*Eco*RV fragment of GSK-3 β (nucleotide 47–3' polylinker) were cloned into the *Bgl*II site of pSP64T (Melton *et al.*, 1984). These fragments contained the entire open reading frames of the two cDNAs. Capped RNA was transcribed from *Xba*I-linearized plasmid with SP6 RNA polymerase and 1 µg used to program a message-dependent rabbit reticulocyte lysate (Promega) in the presence of 100 µCi [³⁵S]methionine. After incubation for 1 h at 30°C, the proteins were denatured by heating at 95°C in 1% SDS, resolved on a 15% polyacrylamide gel and radioactivity detected by fluorography.

Transfection of COS-1 cells

The same DNA fragments of GSK-3 α and β used for *in vitro* transcription were ligated into the *Eco*RI site of pmt-2 (Wong *et al.*, 1985). Plasmid DNA (20 µg) was transfected into two 6 mm dishes of COS-1 cells by calcium phosphate precipitation in the presence of chloroquine (Luthman and Magnusson, 1983) or by lipofection (Gibco) according to the supplier. Cells were washed with ice-cold Tris-buffered saline 48 h after introduction of DNA and lysed at 4°C in 20 mM HEPES-OH, 1 mM EDTA, 1 mM dithiothreitol (buffer A) containing 1% Triton X-100 and 10 mM NaCl. After brief sonication, the lysate was centrifuged at 10 000 g for 30 min and the supernatant applied to a monoS FPLC column equilibrated in buffer A containing 0.05% Triton X-100. Proteins were eluted with a 20 ml 0–500 mM NaCl gradient. Fractions were assayed for glycogen synthase kinase activity as described (Woodgett and Cohen, 1984).

Partial purification of GSK-3 from bovine brain

The procedure is modified from Tung and Reed (1989). All procedures were performed at 4°C. Briefly, fresh bovine brain (500 g) was homogenized in 1 l of 50 mM Tris–HCl, 4 mM EDTA, 2 mM EGTA, 15 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM benzamide, pH 7.5 and centrifuged at 6000 g for 40 min. The supernatant was passed through DEAE–Sephacryl FF (5 \times 30 cm) and the flow-through fractions directly applied to CM–Sephacryl FF (both columns equilibrated in 25 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 15 mM 2-mercaptoethanol, 5% glycerol, pH 7.5; buffer A). GSK-3 was eluted with buffer A + 200 mM NaCl. The active fractions were diluted 2-fold and applied to phosphocellulose (7 \times 7 cm) in buffer A. After washing with buffer A + 100 mM NaCl, GSK-3 was eluted with a 100–500 mM NaCl gradient (Figure 4A). The active fractions were dialysed against buffer A and applied to heparin–Sephacryl (2.6 \times 5 cm) and eluted with a gradient of 100–500 mM NaCl (Figure 4B). Active fractions were dialysed against buffer A and concentrated by chromatography on S–Sephacryl (1 \times 10 cm), eluting with buffer A + 250 mM NaCl. The protein peak was size fractionated by Sephacryl S-300HR (2.6 \times 90 cm) in buffer A + 200 mM NaCl (Figure 4C). The activity eluted in a symmetrical peak corresponding to 70 kd. The active fractions were concentrated and dialysed against buffer A containing 200 mM NaCl and 50% glycerol and stored at -20°C . The preparation had a specific activity of 0.35 µmol phosphate transferred into glycogen synthase per min per mg at 30°C.

Immunological methods

The C-terminal regions of the two forms of GSK-3 were fused to TrpE or glutathione S-transferase for bacterial expression. Briefly, for TrpE fusions: a 660 bp *Clal* fragment of GSK-3 α extending from amino acid 302 to beyond the C-terminus was cloned into the unique *Clal* site of pATH-3 (constructed by T.J.Koerner and A.Tzagaloff; Angel *et al.*, 1988); a 750 bp *Bam*HI fragment of GSK-3 β extending from amino acid 189 to beyond the C-terminus was ligated into the unique *Bam*HI site of pATH-2. The resulting plasmids, pATH α and pATH β respectively, were transformed into C600 bacteria. TrpE fusion proteins of 53 kd (pATH α) and 58 kd (pATH β) were expressed and purified from inclusion bodies by standard procedures (Sambrook *et al.*, 1989). For glutathione S-transferase fusions: a *Pvu*II fragment of GSK-3 α from amino acid 306 to the C-terminus was filled in and ligated into the *Sma*I site of pGEX1 (Smith and Johnson, 1988) to form pGEX α ; a 750 bp *Bam*HI fragment of GSK-3 β amino acid 189 to C-terminus was ligated into the *Bam*HI site of pGEX1 to form pGEX β . Fusion proteins of 45 kd (pGEX α) and 50 kd (pGEX β) were purified by chromatography on glutathione–agarose (Pharmacia) (Smith and Johnson, 1988). The two TrpE fusion proteins (200 µg) were separately mixed with Freund's adjuvant and each subcutaneously injected into two rabbits. The animals were boosted every 4–6 weeks and serum collected after 7 days. The glutathione S-transferase fusion proteins were used for antisera characterization and quantitation. It should be noted that although the regions of the two proteins used as antigens contained stretches of highly homologous peptide sequence (Figure 1C), the antibodies raised were specific for the

respective antigen, especially in later bleeds. Presumably the common peptides are less antigenic than the distinct peptides. The antibodies recognized denatured and, with lower efficiency, native GSK-3 protein.

For immunoblotting, various rat tissues were homogenized on ice in 4 volumes of 20 mM HEPES-OH, 1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, pH 7.5 using a Polytron cell disrupter. An equal volume of 2× Laemmli sample buffer (Laemmli, 1970) was added and the samples boiled for 5 min. After brief sonication, tissue extracts (100 µg) were separated on 12.5% polyacrylamide gels and electrophoretically transferred to PVDF membranes (Millipore). Blots were processed using standard procedures (Harlow and Lane, 1988); antiserum passed through DEAE–Affigel Blue (Bio-Rad) was used at 50- to 100-fold dilutions and detection was with [¹²⁵I]protein A (Amersham).

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Note added in proof

The nucleotide sequence data reported here will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X53427 (GSK-3α) and X53428 (GSK-3β).